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(57) Abstract

The invention relates to a PHB-producing microorganism which grows and produces PHB on or in minimal medium comprising only glycerol as source of carbon and energy and which produces PHB in industrially attractive quantities on or in a medium containing at least glycerol as source of carbon and energy. In particular, a PHB-producing microorganism which is a mutant of a PHB-producing microorganism which cannot be grown on glycerol and belongs to the species *Alcaligenes eutrophus*. The invention also relates to a method of obtaining such a PHB-producing microorganism according to the invention and to a method of producing a PHB, in which such a PHB-producing microorganism according to the invention is subjected to fermentation with a nutrient medium comprising at least glycerol as source of carbon and energy under conditions which are such that the microorganism accumulates PHB and the PHB thus formed is extracted in a manner known per se. Furthermore, the invention also relates to a method of removing glycerol from a glycerol-containing medium using a PHB-producing microorganism according to the invention. In particular, of removing glycerol from a glycerol-containing medium originating from a method of producing biodiesel.

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PHB-PRODUCING MICROORGANISM AND PROCESS FOR REMOVING GLYCEROL FROM A CULTURE MEDIUM.

The invention relates to a PHB-producing microorganism, a method of obtaining a PHB-producing microorganism, a method of producing PHB and a method of removing glycerol from a glycerol-containing medium.

Poly 3-hydroxyalkanoate (PHA) is a collective name for biologically degradable polyesters which are accumulated intracellularly as a reserve material by many bacteria. These optically active polyesters are biocompatible and biodegradable, and they have properties which vary with their composition. Because of the interesting properties and various possible applications, the industrial interest in PHAs has increased considerably in recent years. A number of potential applications for PHAs are mentioned below:

1. Replacement of petrochemically produced plastics, for example packaging materials. PHAs are fully biologically degradable and are produced from raw materials which replenish themselves again and are therefore in principle inexhaustible.
2. PHAs are not only biodegradable, but also biocompatible, i.e. they do not induce any immune reactions in the body. These properties make PHAs suitable in principle for medical applications.
3. The monomers in PHA are identical stereoisomers and are therefore interesting as starting materials for the synthesis of chiral compounds.

Of the PHAs, polyhydroxybutyrate (PHB) is one of the best known (Lemoigne, 1926). PHB is accumulated by many different types of bacteria during unbalanced growth on substrates such as sugars, ethanol and methanol. PHB is a very crystalline thermoplastic, while other known PHAs generally contain monomers having a medium-long chain length and are elastomers having low melting points (Marchessault et al, 1990).

PHBs, and in particular the copolymer PHB/HV (polyhydroxybutyrate-co-hydroxyvalerate) can be processed by conventional techniques to produce small bottles, films, fibres etc. The mechanical properties of these polymers are best compared with those of polypropylene, but PHBs are not gas-permeable.

In the past decade, many genetic and biochemical investigations have been carried out on PHB production. It has been found that PHB is synthesized from acetyl-coA via a three-stage biosynthetic route (Anderson and Dawes, 1990). In addition to 3-hydroxybutyrate, a number of other monomers, such as 3-hydroxyvalerate, 4-hydroxybutyrate and 5-hydroxyvalerate, can be incorporated in this polymer (Doi et al., 1987; 1990).

A large amount of investigation has been carried out into various combinations of microorganisms and nutrient media for the production of PHB on an industrial scale. It was found that Alcaligenes species were the best production organisms, while carbohydrates formed the most suitable substrates in view of availability, price, fermentation technology, PHB yield and PHB quality (Byrom, 1987; Hanggi, 1990). In 1990, Imperial Chemical Industries (ICI) started commercial production of the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) P(HB-co-HV) using a fermentation process which comprised two stages. In the first stage, cells were cultured on glucose. In the second phase, PHB was accumulated in the bacteria cell to an amount of 70-80% of the dry weight under the influence of a limitation, for example a nitrogen or phosphorus limitation. In this stage, both glucose and propionic acid were administered. It was found that the ratio of propionic acid to glucose determines the 3-hydroxyvalerate content of the polymer. The polymer composition of the product can therefore be varied in a controlled manner by adjusting the substrate mixture. The copolymer PHB/HV has advantages over the homopolymer PHB in that the melting point is lower, it is less crystalline, it is less brittle and it has better elastic properties. PHB/HV is obtainable under the name Biopol. EP-B-0,046,344 describes a fermentation method of producing PHB by aerobic culturing of a micro-organism of the Alcaligenes genus, in which method at least one of the requirements needed for growth but not for accumulation is not fulfilled so that the bacterial cells contain at least 25% PHB. The amount of nitrogen or phosphorus in the medium can be limited to achieve this. This has the disadvantage that the rate of PHB synthesis is not optimal.

The Austrian company BTF has developed a fermentation process in which PHB can be produced on the basis of sucrose using the bacterial strain Alcaligenes latus. This process has functioned successfully on a pilot scale. EP-B-0,144,017 describes a fermentation method in which Alcaligenes latus is used for producing PHB instead of Alcaligenes

eutrophus because Alcaligenes latus accumulates PHB without limitation of nutrients such as nitrogen and phosphorus, as a result of which the rate of PHB formation can remain optimal during accumulation, with the result that continuous culturing is possible. However, an oxygen limitation is required for Alcaligenes latus during the accumulation stage. An advantage of the use of Alcaligenes latus over Alcaligenes eutrophus should be the greater thermal tolerance, which makes culturing at higher temperature possible. However, PHB accumulation and utilization of a carbon source are better with Alcaligenes eutrophus.

In an ATO-DLO report by Eggink et al. (1992), Ind. Crops + Products (in press), a first analysis is given of the potential use of long-chain fatty acids (LCFAs) as nutrients for PHB fermentations. It was found that the PHB yield of A. eutrophus on oleic acid is twice as high as the yield with carbohydrates. Both the molecular weight and the melting point of the PHB which is produced from oleic acid are comparable with the values found for PHBs formed from carbohydrates. This method must be investigated further since PHB yield is only one of the factors which determines the production time of the fermentation. The rate of product formation and product concentration are also important. In addition, the use of LCFAs entails specific problems in fermentations, such as a high oxygen requirement and insufficient mass transfer because of the system, which is composed of two liquid phases. Initial results show that, if the oleic acid concentration is maintained at 0.5% (weight/volume) cell dry weights of 50 g/l comprising 65% PHB can be obtained within 60 hours after the start of fermentation. In addition, the possibility of synthesizing a copolymer containing 3-hydroxyvalerate has also been investigated by using a nutrient medium containing nonanoic acid in addition to oleic acid.

In the same paper, the use of oleic acid as substrate for PHA formation by P. putida was also investigated. This investigation has shown that a number of new PHAs can be produced by varying the fatty acid composition of the medium. At present, the β oxidation and PHA synthesis by P. putida is investigated during culturing on fatty acids such as linoleic acid, petroselinic acid, ricinoleic acid and vernolic acid. No specific applications are as yet known for the PHAs which are produced by Pseudomonas strains, and these polymers have hitherto been produced and investigated only on a laboratory scale.

From economic analyses, it is evident that the commercial success and the final market share of PHB and PHB/HV will depend to a significant extent on the price. In a large-scale production, the raw material costs will form an appreciable proportion (30%) of the total production costs of PHB. Thus, approximately 3.5 kg of glucose or sucrose is necessary to produce 1 kg of PHB. The price per kilo of these sugars is approximately 60 to 80 cents. Cheaper production methods, for example biosynthesis in transgenic plants, and cheaper raw materials are therefore being sought on a worldwide basis. In the process in which LCFAs originating from vegetable oils are used, it has been found that 1.5-2 kg of fatty acids can yield 1 kg of PHB if A. eutrophus is cultured.

Hitherto, the industrial process of ICI appears, however, to be the most suitable for large-scale production of PHB. This is because of the fact that the microorganism A. eutrophus can be handled easily, grows well on minimal salt media, grows rapidly to high cell densities and accumulates PHB up to approximately 80% of the dry weight and does not secrete any undesirable metabolites in doing so. In addition, the PHB polymers can be extracted satisfactorily from the biomass and the polymer produced has good properties, such as a high molecular weight. The disadvantage of A. eutrophus is, however, that the substrate range is very limited. Cheap agricultural products, such as, for example, starch and sucrose, which are available in large quantities cannot be used as raw material in fermentations employing A. eutrophus.

There is therefore a demand for a microorganism which can be used in a method of producing PHB, in which the product can be produced rapidly and in large quantities, the product is also easy to extract, and a cheap source of carbon and energy can be used in the fermentation medium.

Plans are well advanced in the EC and the Netherlands to use vegetable oils, including rapeseed oil, on a large scale as engine fuel. This so-called biodiesel is already being produced and used at present on a small scale. Biodiesel has the advantages that

1) it is produced from raw materials which replenish themselves again,
2) it contains no harmful substances,
3) it is completely biologically degradable, and
4) it forms a new, extensive sales market for vegetable oil.

The vegetable oils are not used directly as fuel but are first converted into methylated fatty acids. In this process, an appreciable quantity of glycerol is formed (approximately 1 kg of glycerol per 10 kg of vegetable oil). Until now the sale of glycerol which is produced by the oleochemical industry has not been a problem, but a large-scale production of biodiesel will certainly result in an excess of glycerol. From an economic and environmental point of view it is therefore of great importance that new, environmentally friendly industrial applications are developed for glycerol.

Bergey's Manual of Systematic Bacteriology (1984) describes how a large number of different bacteria can grow on glycerol as the sole form of carbon and energy. Hitherto no Alcaligenes eutrophus PHB-producing microorganism which is able to grow on glycerol as the sole form of carbon and energy and can produce PHB in industrially attractive quantities has been known. A person skilled in the art knows what requirements have to be fulfilled in order to be industrially attractive. In all cases, the yield must be comparable to or better than the existing PHB producers which are used industrially.

The present invention, which aims to solve the above problems, relates to a PHB-producing mutant of Alcaligenes eutrophus which grows and produces PHB on or in a minimal medium containing only glycerol as source of carbon and energy and which produces PHB in industrially attractive quantities on or in a medium containing at least glycerol as source of carbon and energy. Industrially attractive quantities are preferably understood as meaning that the cell may comprise at least 50% PHB. Furthermore, an industrially attractive microorganism is preferably not only capable of achieving high PHB concentrations but can relatively easily be cultured to a high biomass concentration.

A PHB-producing microorganism has been found which is a mutant of the species Alcaligenes eutrophus, a species which does not grow on glycerol, while the mutant does grow and produces PHB in minimal medium comprising only glycerol as source of carbon and energy.

In particular, it has been found that a PHB-producing microorganism which grows on or in minimal medium comprising only glycerol as source of carbon and energy and which belongs to the variant Alcaligenes eutrophus H16 is eminently satisfactory.

The present invention also relates to a method of obtaining a PHB-producing microorganism which grows on or in minimal medium

comprising only glycerol as source of carbon and energy and produces PHB in industrially attractive quantities on or in a medium comprising at least glycerol as source of carbon and energy. This method is characterized in that a PHB-producing microorganism which cannot metabolize glycerol is placed as starting material on or in a medium comprising only glycerol as source of carbon and energy and it is then cultured, the new microorganisms produced in the course of time being isolated from or out of said culture and being investigated in a manner known per se for PHB production and the microorganisms which are thus obtained and which produce PHB being isolated. In this method, at least one duplication time of the PHB-producing microorganism which is used as starting material and which is unable to metabolize glycerol must have elapsed before the new microorganisms are isolated. Preferably, this isolation takes place after 10 duplication times and still more preferably after 20 duplication times. Preferably, an Alcaligenes eutrophus is used as starting strain for the selection method.

The present invention also relates to a method of producing PHB, in which a PHB-producing microorganism is subjected to fermentation using a nutrient medium comprising at least one source of carbon and energy under conditions which are such that the microorganism accumulates PHB and the PHB formed in this way is extracted in a manner known per se, which method is characterized in that a microorganism is selected as PHB-producing microorganism which is a mutant of a microorganism which cannot grow and cannot produce any PHB with a nutrient medium comprising glycerol as sole source of carbon and energy, while the mutant does grow and produces PHB under these circumstances, the selected organism for accumulation of PHB being subjected to fermentation on or in a nutrient comprising at least glycerol as source of carbon and energy. A good strain is an Alcaligenes eutrophus mutant which can grow and accumulate PHB on glycerol.

In order to achieve accumulation of PHB in the microorganism according to the invention, the fermentation can be carried out under nutrient-limiting conditions, the carbon source not, however, being limited. The fermentation can, for example, be carried out using a nutrient medium which comprises at least one of the nutrients from the group comprising nitrogen, oxygen, phosphorus, sulphur, potassium and magnesium in a limiting quantity. This method is in principle suitable for fed-batch culturing.

To obtain a rapid growth of the PHB-producing microorganism in a method according to the present invention, the fermentation is preferably carried out with a nutrient medium also comprising one or more compounds from the group comprising fatty acids, methylated fatty acids, ethanol, fructose, glucose and acetate in addition to glycerol.

After sufficient growth has been effected, the medium in which culturing is carried out may be altered in such a way that at least one nutrient is present in a limiting quantity, as described above. As a result, PHB accumulation will take place in the microorganism. It is also possible that the method of producing PHB proceeds in the first instance from a medium containing no glycerol for the growth phase followed by a change-over to a medium comprising glycerol and also a nutrient other than carbon as limiting factor.

Another suitable embodiment of the method of PHB production according to the present invention comprises culturing the selected microorganism on glycerol, cell growth and PHB accumulation taking place simultaneously. A continuous culturing yields the PHB production. In particular, this is possible if a glycerol-positive mutant of Alcaligenes eutrophus is used in continuous culturing on a medium comprising glycerol as carbon source. Preferably, no nutrient limitation is applied during continuous culturing.

As a result of culturing on glycerol, PHB can be produced in a simple one-stage fed-batch culturing or in continuous culturing. PHB accumulation greater than 80% can be achieved by the present method. In particular, the Alcaligenes eutrophus mutant GE1 (DSM 7237) is capable of growing on glycerol as sole form of carbon and energy. In addition, GE1 PHB accumulates during the growth. In the case of experiments in 50 ml cultures in which nitrogen had a limiting action for further growth as a result of an excess of glycerol, PHB contents of 80% of the dry weight were easily reached.

It is possible to vary the composition of the PHB produced by providing the composition of medium in which the fermentation takes place with one or more constituents comprising valerate and/or propionate groups during the accumulation phase.

Furthermore, the method according to the invention can be optimized by optimizing any potential limiting factors other than nutrients, such as the substance transfer and oxygen transfer and other processes which result in a lower metabolic rate at high cell densities.

The invention also relates to a method of removing glycerol from a glycerol-containing medium, which method is characterized in that a PHB-producing microorganism according to the invention as described above is used in a fermentation process with the medium containing the glycerol to be treated.

In particular, this method is suitable for treating a glycerol-containing medium originating from a method for the production of biodiesel. As has already been stated, a medium is used for producing biodiesel which comprises methylated fatty acids. It is therefore preferable that a PHB-producing microorganism is used which is able to use not only glycerol but also methylated fatty acids as source of carbon and energy. A very suitable example of this is a microorganism belonging to the species Alcaligenes eutrophus, in particular an Alcaligenes eutrophus H16 variant which is glycerol-positive, such as the strain GE1 (deposited at DSM in Braunschweig on 15 September 1992 under No. DSM 7237).

The invention is further illustrated with the aid of the examples below.

Example I

Selection of A. eutrophus H16 mutants.

In the experiments described here, use has been made of Alcaligenes eutrophus H16 (DSM 428, deposited in the Deutsche Sammlung für Mikroorganismen [German Microorganism Collection] in Braunschweig), which grows optimally at 30°C. As a result of selection on minimal E2 agar plates containing 1% (weight/weight) of glycerol, A. eutrophus H16 variants have been found and isolated which are able to use glycerol as sole form of carbon and energy. Said variants, referred to below as A. eutrophus GE1, appeared after 4 days incubation on minimal plates. On transferring these variants to a new minimal plate, incubation for one night was sufficient to observe growth and colony formation. The E2 medium used consists of 3.5 g of $NaNH_4HPO_4.4H_2O$, 7.5 g of $K_2HPO_4.3H_2O$, 3.7 g of KH_2PO_4, 1 mM of $MgSO_4.7H_2O$, 2.78 mg of $FeSO_4.7H_2O$, 1.98 mg of $MnCl_2.4H_2O$, 2.81 mg of $CoSO_4.7H_2O$, 1.47 mg of $CaCl_2.2H_2O$, 0.17 mg of $CuCl_2.2H_2O$ and 0.29 mg of $ZnSO_4.7H_2O$ per litre.

Growth of A. eutrophus GE1 on glycerol.

A. eutrophus GE1 has furthermore been tested in liquid cultures (50 ml on E2 medium and 1% (weight/weight) glycerol as source of carbon and energy). The duplication time was 4-5 hours. As a comparison, the duplication time for other substrates, such as fructose and LCFAs, is approximately 2 hours.

Example II

PHB accumulation by A. eutrophus GE1

During growth of the A. eutrophus GE1 in 50 ml cultures containing 0.5 E2 medium and 1% glycerol, PHB was accumulated to 85% of the dry weight under the influence of a nitrogen limitation. 0.5 E2 medium contains half the amount of nitrogen and phosphate-containing salts which are present in E2 medium.

Isolation and structural analysis of PHB

PHB was extracted for 4 hours using chloroform in a soxhlet apparatus from freeze-dried cells. After cooling, the extract was evaporated down using a film evaporator. The polymer was redissolved in 15 ml of chloroform and precipitated by adding 150 ml of methanol. The precipitated polymer was dried in air.

The monomer composition of the polymer was determined with the aid of gas chromatography and NMR analysis. For the gas-chromatographic analysis, the polymer was dissolved in 2 ml of chloroform to which 2 ml of 15% sulphuric acid in methanol was added. The mixture was heated to 100°C in a closed container for 140 minutes. After cooling, 1 ml of demineralized water was added to the sample. After phase separation, the organic phase was collected and then dried by adding sodium sulphate. The methyl esters were analyzed using a Carlo Erba gas chromatograph 6000 equipped with a CP-Sil 5CB column. A mixture of bacterial fatty acid methyl esters (Supolco Inc., Bellefonte, United States of America) was used to identify the peaks. With this analysis method it was determined that the polymer formed by A. eutrophus GE1 under the conditions already described consists of more than 99% 3-hydroxybutyrate and less than 1% 3-hydroxyvalerate.

The structure of the polymer has furthermore been analyzed with the aid of 1H and 13C NMR. The polymer was dissolved in deuterated chloroform. The spectra have been recorded on a Bruker AMX 400wb

spectrometer with a probe temperature of 27°C. The NMR analyses have confirmed that the polymer is a polyester based on 3-hydroxybutyrate.

Example III

PHB accumulation by A. eutrophus GE1

A. eutrophus GE1 has also been cultured in 2-litre fermenters in which temperature, pH and dissolved oxygen were kept constant. All the necessary nutrients were administered in a fed-batch process so that none of the nutrients were limiting. During such a process, cell densities of 35 grams dry weight were reached. In the case of such a process, the PHB content was 70% for a cell density of 5 grams per litre. For the final cell density of 35 grams per litre, the PHB content was 80%. Without nutrient limitation, GE1 is able to accumulate up to 80% of the dry weight on glycerol as carbon source. The combination of A. eutrophus mutant and glycerol is eminently suitable for production in continuous cultures because growth and PHB accumulation to very high contents occur simultaneously.

Claims

1. PHB-producing microorganism which is a mutant of a PHB-producing microorganism which cannot be grown on glycerol and belongs to the species Alcaligenes eutrophus, which mutant grows and produces PHB on or in minimal medium comprising only glycerol as source of carbon and energy and produces PHB in industrially attractive quantities on or in a medium comprising at least glycerol as source of carbon and energy.

2. PHB-producing microorganism according to Claim 1, which is a mutant of a PHB-producing microorganism belonging to the variant Alcaligenes eutrophus H16 which cannot be grown on glycerol.

3. PHB-producing microorganism according to Claim 2, which is the mutant GE1 deposited at DSM in Braunschweig on 15 September 1992 under No. DSM 7237.

4. Method of obtaining a PHB-producing microorganism, in which a PHB-producing microorganism which cannot metabolize glycerol, preferably an Alcaligenes eutrophus, is placed on or in a medium comprising only glycerol as source of carbon and energy and is then cultured, new microorganisms produced in the course of time being isolated from or out of said culture and being investigated in a manner known per se for PHB production and the mutant microorganisms which are thus obtained and which are capable of producing industrially attractive quantities of PHB and are able to grow on only glycerol as source of carbon and energy being isolated.

5. Method according to Claim 4, in which the mutant microorganisms are isolated after at least 10 duplication times of the PHB-producing microorganism which is used as starting material and which cannot grow on only glycerol as source of carbon and energy and cannot produce PHB in industrially attractive quantities under these circumstances.

6. Method of producing PHB, in which a PHB-producing microorganism is subjected to fermentation with a nutrient medium comprising at least one source of carbon and energy under circumstances which are such that the microorganism accumulates PHB, and the PHB formed in this way is extracted in a manner known per se, a mutant according to one of Claims 1-3 or a microorganism obtainable according to Claim 4 or 5 being subjected to fermentation in order to accumulate PHB on or in a nutrient medium which comprises at least glycerol as source of carbon and energy.

7. Method according to Claim 6, in which, in order to accumulate PHB, the fermentation is carried out under nutrient-limiting circumstances, the carbon source not being limited.

8. Method according to Claim 6 or 7, in which, to accumulate PHB, the fermentation is carried out using a nutrient medium which comprises at least one of the nutrients from the group comprising nitrogen, oxygen, phosphorus, sulphur, potassium and magnesium in a limiting quantity.

9. Method according to Claim 6, in which both cell growth to a sufficiently high biomass and accumulation of PHB are carried out in a one-stage culture of the PHB-producing microorganism.

10. Method according to Claim 9, in which PHB is produced in a continuous culture.

11. Method according to Claim 9 or 10, in which culturing is carried out under conditions which are such that no nutrient-limiting circumstances occur.

12. Method according to one of Claims 6-11, in which the fermentation is carried out using a nutrient medium which also comprises one or more compounds from the group comprising fatty acids, methylated fatty acids, ethanol, fructose, glucose and acetate.

13. Method according to one of Claims 6-12, in which, in order to accumulate PHB, the fermentation is carried out using a nutrient medium also comprising one or more constituents containing valerate and/or propionate groups.

14. Method according to one of Claims 6-13, in which, in order to accumulate PHB, the fermentation is carried out using a nutrient medium originating from the production of biodiesel.

15. Method according to one of Claims 6-14, in which the fermentation is carried out using a PHB-producing mutant according to one of Claims 1-3 and/or using a PHB-producing microorganism obtainable via the method according to Claim 4 or 5.

16. Method of removing glycerol from a glycerol-containing medium, in which a PHB-producing mutant according to one of Claims 1-3 and/or a microorganism obtainable via the method according to Claim 4 or 5 is used in a fermentation process with the medium containing the glycerol to be treated.

17. Method according to Claim 16, in which the medium containing the glycerol to be treated originates from a method for the production of biodiesel.

18. Method according to Claim 16 or 17, in which a PHB-producing microorganism is used which is also able to use methylated fatty acids as source of carbon and energy.

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/NL 93/00205

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12P7/62 C12N1/32 C08G63/06 C12N1/20 //(C12N1/20, C12R1:05)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12P C12N C08G C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 144 017 (CHEMIE LINZ AKTIENGESELLSCHAFT) 12 June 1985 see page 1 - page 12 see claims	1,6,16
X	FR,A,2 360 667 (AGROFERM) 3 March 1978 see page 6 see claims	1,6,16

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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